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Research Article

Cyclin D1 Overexpression Predicts Poor Disease-Specific Survival in Human Papillomavirus-Independent Vulvar Squamous Cell Carcinoma

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ABSTRACT

The amplification of *CCND1* is associated with the development and progression of various cancers. In a recent study, we showed that almost all adverse outcomes in vulvar squamous cell carcinomas (VSCC) occurred in patients with human papillomavirus (HPV)−independent, *TP53*-mutated tumors harboring *CCND1* gains. In this study, we analyzed the association between *CCND1* gain, cyclin D1 immunohistochemistry (IHC), and disease-specific survival (DSS) in a series of patients with HPV-independent VSCC. All patients who underwent primary surgery for VSCC at the Hospital Clínic of Barcelona, Spain, from 1975 to 2023 were recruited ("overall" cohort, $n = 139$). IHC for p53 and cyclin D1 was performed in all cases. In a subset of patients, we performed DNA sequencing to evaluate *CCND1* copy number variations ("sequencing" cohort, $n = 54$). Cyclin D1 IHC overexpression ($\geq$50% of tumor cells) had 94% sensitivity and 67% specificity as a surrogate marker of *CCND1* gain. In the "sequencing" cohort, only *CCND1* gains were significantly associated with impaired DSS in the multivariate analysis (hazard ratio [HR], 4.15; 95% CI, 1.08-5.40; $P = .032$), whereas stage or mutant *TP53* status did not reach statistical significance. In the "overall" cohort, advanced stage (HR, 2.41; 95% CI, 1.08-5.39; $P = .032$) and cyclin D1 IHC overexpression (HR, 4.89; 95% CI, 1.77-18.5; $P = .001$) were associated with worse DSS in the multivariate analysis, whereas abnormal p53 IHC was not (HR, 5.06; 95% CI, 0.68-647; $P = .138$). In conclusion, cyclin D1 overexpression is an acceptable surrogate for *CCND1* gain and has a much stronger adverse prognostic impact than altered p53 IHC in patients with HPV-independent VSCC.

Introduction

Vulvar squamous cell carcinoma (VSCC) is a rare cancer that develops via 2 different pathways: human papillomavirus (HPV)-associated and HPV-independent.[1] HPV-associated VSCC affects younger women and is characterized by immunohistochemical

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(IHC) overexpression of p16, a surrogate biomarker of HPV infection.[2] HPV-independent VSCC, which comprises the vast majority of VSCC cases in high-income countries, affects older women and arises in a background of chronic vulvar inflammatory lesions.[3] There is compelling evidence indicating that HPV-independent tumors should be further subclassified according to the *TP53* status as *TP53* mutant and *TP53* wild-type. Increasing evidence indicates that the prognosis of HPV-independent VSCC is poor,[2,4,5] and that HPV-independent VSCC with *TP53* mutation or abnormal p53 IHC are associated with a particularly poor prognosis.[6-10] No genomic alterations other than *TP53* or p53 IHC abnormalities have been consistently described as prognostic factors in HPV-independent VSCC.[7,11,12]

Cyclin D1, a protein encoded by the *CCND1* gene located at 11q13,[13] is a well-known key player in cell cycle regulation through promotion of the G1-S phase.[14,15] Cyclin D1 overexpression triggers the activation of cyclin-dependent kinases and thereby promotes cell cycle G1/S transition, a phenomenon involved in the development and progression of many cancers, and the *CCND1* gene is one of the most commonly amplified loci in solid cancers,[16] including VSCC.[17] Amplification of *CCND1* is one of the main pathways that leads to cyclin D1 overexpression.[18] Several studies have shown both cyclin D1 expression[19-21] and *CCND1* gains[17,20,22,23] in VSCC, confirming the presence of these cell cycle alterations in this cancer. Cyclin D1 overexpression has been shown to be more frequent in HPV-independent than in HPV-associated VSCC.[17,19,20]

The possible relationship between cyclin D1 overexpression and prognosis has been addressed in very few studies, and only 1 study has explored the correlation between *CCND1* copy number variation and protein (cyclin D1) expression in VSCC.[20] Although a correlation between cyclin D1 overexpression and lymph node metastases has been found,[20] other reports have failed to show a direct prognostic association.[20,21] In a recent study, we showed that *CCND1* gains were almost exclusively identified in HPV-independent VSCC and were associated with an adverse prognosis.[24] However, owing to the limited number of cases included in this latter study and the scarcity of reported series on this topic,[20,21] no reliable conclusions on the prognostic value of cyclin D1 expression could be drawn.

In this study, we aimed to explore the association between *CCND1* gains and cyclin D1 expression levels and correlate cyclin D1 overexpression with clinical and pathological parameters and prognosis in a large series of patients with HPV-independent VSCC.

Methods

Patients

We retrospectively identified all patients who underwent primary surgery for VSCC at the Department of Gynecological Oncology of the Hospital Clínic of Barcelona, Spain, over a 48-year period (February 1975-February 2023). The following clinicopathological variables were retrieved from the electronic archives: patient age at diagnosis, type and date/s of treatment/s, pathological features of the tumor (size, location, depth of invasion, unifocality/multifocality, margin status, and lymph node involvement), and follow-up data (date and site of cancer recurrence, if any; date and cause of death). All available pathological materials for the cases were reviewed by 2 gynecological pathologists with expertise in vulvar pathology (N.R. and A.S.).

This study included 2 cohorts of patients. The "overall" cohort included all patients diagnosed with HPV-independent VSCC in the above-mentioned 48-year period who fulfilled the following inclusion criteria: (1) pathological diagnosis of VSCC treated primarily with surgery and (2) sufficient tumor tissue available for IHC analysis (p16, p53, and cyclin D1) and HPV DNA testing. The following exclusion criteria were established: (1) positive staining for p16 and/or positive HPV DNA testing results; (2) neoadjuvant radiotherapy, neoadjuvant chemotherapy, or palliative primary treatment; and (3) follow-up time shorter than 6 months. Thus, only HPV-independent tumors treated primarily with surgery and with a follow-up of at least 6 months were included in the study.

The second cohort, called the "sequencing" cohort was randomly selected from the "overall" cohort. In addition to the above-mentioned inclusion and exclusion criteria, the cases included in this cohort fulfilled the following inclusion criteria: sufficient material for sequencing analysis, and exclusion criteria were established in the "sequencing" cohort—low coverage depth (<20) in the tumor after receiving sequencing results.

Institutional ethical approval for this study was obtained in 2020 (registry reference: HCB/2020/1198). All participating patients provided written informed consent in accordance with institutional and legal regulations.

DNA Extraction

DNA extraction for sequencing and HPV testing was performed on the tumor area from formalin-fixed paraffin-embedded tissue from surgical VSCC specimens. For the normal tissue specimens, whole sections of selected blocks containing only normal vulvar skin were used. DNA was extracted using the QIAmp DNA Tissue Kit (Qiagen), and quality was controlled using a Qubit dsDNA high-sensitivity assay kit (Thermo Fisher Scientific) and a quality control assay (Roche).

Human Papillomavirus Detection, p16 Immunohistochemistry, and Assignment of Human Papillomavirus Status

SPF10 PCR and the LiPA25 system (version 1; Labo Biomedical Products) were used for HPV DNA detection, and genotyping was performed using an INNO-LiPA HPV Genotyping Extra II kit (Fujirebio).

p16 IHC staining was performed on all samples using a CINtec Histology Kit (clone E6H4; Roche). Tumors with strong and diffuse block-like staining were considered positive, whereas those with completely negative or patchy staining were considered negative.[2]

Cases showing either positive p16 IHC and/or positivity for high-risk HPV were classified as HPV-associated and were, thus, excluded. The categorization of a tumor as HPV-independent required both negative p16 IHC staining and absence of high-risk HPV DNA.

p53 Immunohistochemistry

p53 IHC staining was performed in all cases using a monoclonal antibody (clone DO-7; Dako). IHC staining was evaluated in the invasive tumor following the recent p53 pattern–based interpretation framework,[25,26] which includes 2 major categories: normal, which correlates with a wild-type protein and includes 2 patterns (scattered cells and mid-epithelial) and

abnormal, which correlates with a mutant protein and includes 4 patterns (diffuse overexpression, basal overexpression, cytoplasmic, and null). The spectrum of wild-type patterns has been expanded to include the null-like pattern.[27] However, both the mid-epithelial and the null-like wild-type patterns have been exclusively described in HPV-associated VSCC.[27,28]

Cyclin D1 Immunohistochemistry

Cyclin D1 IHC staining was performed in all cases using a monoclonal antibody (clone SP4; Thermo Fisher Scientific). Nuclear staining of endothelial cells was used as an internal control.

The number of tumor cells with nuclear positivity of any intensity was counted in a hotspot area of 100 viable tumor cells at ×40 magnification and divided by the total number of viable tumor cells in the same area to obtain the percentage of cyclin D1 expression. Highly keratinized areas were excluded from the evaluation. Three pathologists (N.R., A.S., and L.S.) independently scored all cases.

Sequencing Analyses

All sequencing analyses were performed using formalin-fixed paraffin-embedded tissues. Whole exome sequencing (WES) was performed on the tumor and paired normal tissue (normal vulvar skin) from the same patient, as previously reported.[24] Briefly, WES library preparation was performed using the Kappa HyperExome kit (Roche), followed by sequencing on the Illumina Nova Seq 6000 platform in the paired-end mode with a read length of 2 × 151 bp.

In a subset of VSCC samples, DNA sequencing (Oncomine Comprehensive Assay v3 GX; Thermo Fisher Scientific) of only the tumoral tissue was performed. Libraries were automatically generated using the Ion Chef System (Thermo Fisher Scientific).

Bioinformatics Processing of Sequenced Samples

After sequencing, WES reads were mapped to the human genome (hs37d5) using Burrows-Wheeler Alignment with default parameters. Somatic variant calling was performed using GATK v4.1.9.0 Mutect2 and Strelka2 v2.8.3.[29] Copy number variations in WES cases were predicted using Control-FREEC.[30] Bioinformatics processing of samples sequenced with the Oncomine panel was performed according to the institutional protocol.

All *TP53* mutational variants identified were assigned pathogenicity scores based on the ClinVar database.[31] Based on all *TP53* variants identified, overall pathogenicity was assigned for each case; only tumors with at least 1 pathogenic or likely pathogenic variant were considered as *TP53*-mutant (*TP53*-pathogenic).

Determination of the Cyclin D1 Immunohistochemical Expression Threshold

To determine the most appropriate cutoff for defining cyclin D1 IHC overexpression, the performance of different thresholds for cyclin D1 expression was evaluated against the presence of *CCND1* gains (the gold standard). Multiple strategies for interpreting the cyclin D1 IHC results of the 3 pathologists were evaluated: (1) independent assessment of each rater's evaluation, (2) data aggregation across raters, (3) calculation of the mean score of all raters, and (4) meeting or exceeding each specific threshold by at least 2 raters. The choice of cutoff threshold was guided by the balance between sensitivity and specificity, with Youden's index and receiver operating characteristic (ROC) analysis (area under the curve) employed as a measure of overall diagnostic performance. Once the cutoff threshold was defined, a criterion where at least 2 raters met or exceeded the defined threshold was applied to classify the patients according to cyclin D1 IHC expression (normal or overexpressed cyclin D1). The Fleiss Kappa statistic was used to evaluate interobserver agreement.

Treatment and Follow-Up of the Patients Included

All patients were treated according to clinical guidelines at the time of treatment. Surgery involved either radical vulvectomy or radical tumor excision along with lymph node assessment. Inguinal lymphadenectomy was performed until 1998. From 1999 to 2003, inguinal lymphadenectomy and sentinel lymph node biopsy were performed. In 2002, sentinel lymph node biopsy was recognized as a valid method,[32] and since then, it has been the sole staging procedure for unifocal VSCC measuring <4 cm. Patients with positive sentinel lymph nodes underwent ipsilateral inguinofemoral lymphadenectomy. Adjuvant treatments (radiotherapy and chemotherapy) were administered in accordance with clinical guidelines applicable at the time of diagnosis.

Follow-up care comprised physical examination every 4 to 6 months during the first 2 years and annually thereafter. Imaging techniques (resonance imaging, inguinal ultrasound, or computed tomography) were periodically performed in patients with advanced VSCC or when recurrence was suspected.

Statistical Analysis

Statistical analyses were performed using R v.4.4.0 (R Foundation for Statistical Computing). χ^2 test, Fisher exact test, and Wilcoxon rank sum test were used to compare the clinical and histopathological data between the study groups.

The survival end points were recurrence-free survival and disease-specific survival (DSS), which were calculated from the date of primary surgery to the date of the first recurrence or death due to disease, respectively. Median follow-up times and 95% CIs were estimated using the reverse Kaplan-Meier method.

Survival curves were plotted using Kaplan-Meier estimates and compared using the log-rank test. The Cox proportional hazards model with Firth's penalized likelihood was used to assess the association between the survival outcomes and explanatory variables. Univariate, The International Federation of Gynecology and Obstetrics (FIGO) stage-adjusted, and multivariate analyses were performed. Multivariate models included FIGO stage, p53 status (by IHC and sequencing, according to the cohort), and cyclin D1/*CCND1* status (by IHC and sequencing, according to the cohort). Two-sided tests were applied, with statistical significance at an α level of 0.05.

Results

Clinical Characteristics of the Patients and Classification Into 2 Study Cohorts

During the study period, 190 patients with VSCC were identified. Thirty-three patients had HPV-associated VSCC, 5 patients

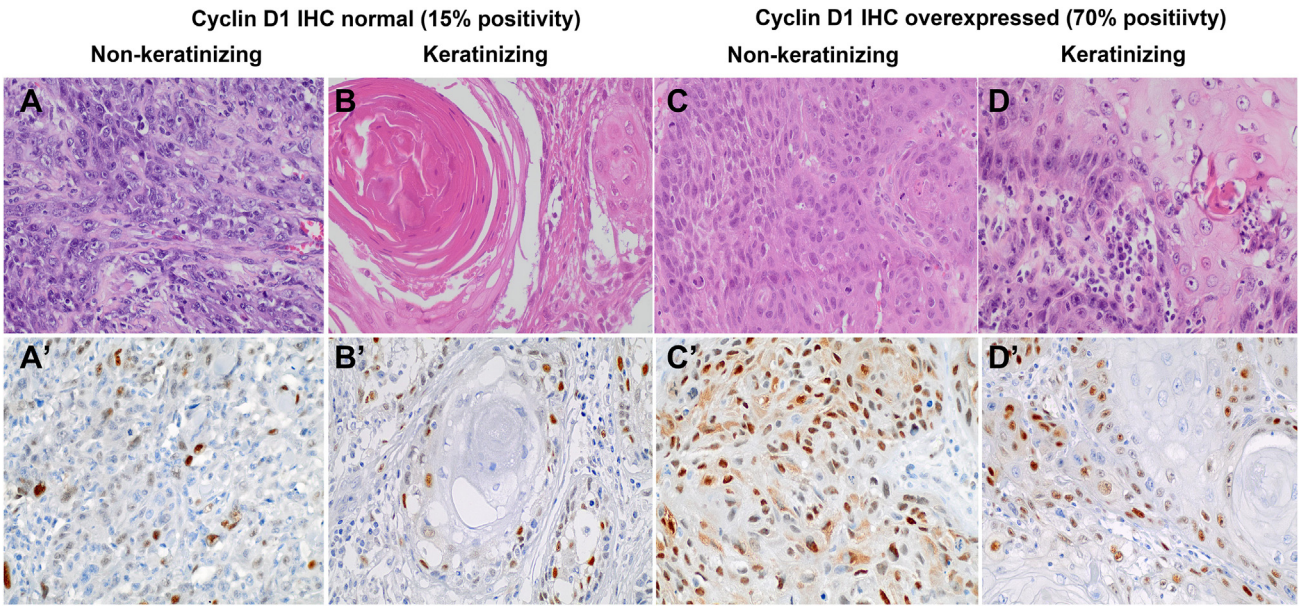

Figure 1.
Example of low (15% positivity) and high (70% positivity) cyclin D1 immunohistochemical expression in nonkeratinizing (A and C) and keratinizing (B and D) vulvar squamous cell carcinomas. IHC, immunohistochemistry.

received neoadjuvant radiotherapy or chemotherapy, 6 underwent palliative treatment, and 7 were lost to follow-up <6 months after surgery; thus, they were excluded from the study. Thus, the "overall" cohort included 139 HPV-independent VSCC cases. Among these, 61 were randomly selected and sequenced. Seven tumors were excluded because of low coverage depth in the final sequencing results. Thus, 54 HPV-independent VSCC were finally included in the "sequencing" cohort.

In the "overall" cohort (139 patients), the median age at diagnosis was 77.7 years (range, 22.6-95.8) and the median follow-up was 60 months. Forty-five of the 139 (32.4%) patients were diagnosed with advanced FIGO stage (stage III or IV). Sixty-two (44.6%) patients were treated with radical vulvectomy, and 77 (55.4%) patients underwent local wide excision. A total of 119 (86.3%) patients underwent surgical lymph node evaluation using a sentinel lymph node biopsy ($n = 47$), inguinofemoral lymphadenectomy ($n = 41$), or both procedures ($n = 31$). Twenty (14.4%) patients did not undergo surgical lymph node evaluation; 4 patients because of FIGO 2021 stage IA (negativity of lymph nodes was assumed) and 16 patients because of poor performance status. Forty-seven (33.8%) patients underwent adjuvant radiotherapy, and 5 (3.6%) patients received adjuvant chemotherapy. The clinical characteristics of the "sequencing" cohort of 54 patients were similar to those of the "overall" cohort, as the former cohort represents a random subsample of the overall cohort.

TP53 Mutational Status and p53 Immunohistochemical Alterations

In the "overall" cohort, 121 of 139 (87.1%) tumors showed abnormal p53 IHC expression: 82 (59.0%) diffuse overexpression staining, 17 (12.2%) null staining, 13 (9.4%) basal overexpression, and 9 (6.5%) cytoplasmic expression. Eighteen (12.9%) patients in the "overall" cohort exhibited normal p53 IHC results, with all presenting a scattered staining pattern.

Forty-one of the 54 tumors from the "sequencing" cohort (75.9%) harbored at least 1 pathogenic or likely pathogenic variant and were classified as *TP53* mutant. Twelve tumors (22.2%) had no *TP53* mutations, and 1 additional tumor (1.85%) carried a *TP53* mutational variant of uncertain significance. Thus, 13 tumors (24.1%) were classified as nonmutant. The p53 IHC results were in agreement with *TP53* mutation status in 46 of 54 cases (85.2%).

Table 1
Diagnostic performance of cyclin D1 immunohistochemistry as a surrogate marker of *CCND1* gains at different cutoff points of expression

Parameter	Cyclin D1 immunohistochemical expression		
	Cutoff 40%	Cutoff 50%	Cutoff 60%
Sensitivity	1 (0.81-1)	0.94 (0.73-1)	0.83 (0.59-0.96)
Specificity	0.39 (0.23-0.47)	0.67 (0.49-0.81)	0.69 (0.52-0.84)
Youden Index	0.39 (0.05-0.57)	0.61 (0.22-0.81)	0.53 (0.10-0.80)
Positive predictive value	0.45 (0.29-0.62)	0.59 (0.39-0.76)	0.58 (0.37-0.77)
Negative predictive value	1 (0.77-1)	0.96 (0.80-1)	0.89 (0.72-0.98)

The values are presented in percentage format along with their corresponding 95% CIs for each cutoff. A tumor was considered overexpressed when at least 2 observers determined that cyclin D1 IHC expression exceeded the respective cutoff value.
The 50% cutoff finally selected as threshold for cyclin D1 overexpression.

Table 2
Agreement between the presence of *CCND1* copy number gains (identified by sequencing) and cyclin D1 overexpression assessed by immunohistochemistry in the 54 patients in the "sequencing" cohort, using a cutoff threshold of at least 2 raters scoring ≥50% of positive tumor cells

Cyclin D1 IHC result	*CCND1* copy number status		Total
	Normal (absence of gains)	Abnormal (presence of gains)	
Normal (<50%)	24 (44%)	1 (1.9%)	25 (46%)
Overexpression (≥50%)	12 (22%)	17 (31%)	29 (54%)
Total	36 (67%)	18 (33%)	54 (100%)

$P < .001$ (χ^2 test).
IHC, immunohistochemistry.

CCND1 Gains, Definition of the Cyclin D1 Immunohistochemical Cutoff, and Correlation With Cyclin D1 Immunohistochemical Overexpression

Cyclin D1 nuclear staining of endothelial cells was detected in all the cases. The percentage of tumor cell expression ranged from 0% to 90%. Examples of low and high cyclin D1 IHC expression in nonkeratinizing and keratinizing tumors are shown in Figure 1. In the "sequencing" cohort, *CCND1* gains were identified in 18 of 54 VSCC (33.0%) cases.

Table 1 shows the diagnostic performance of cyclin D1 IHC as a surrogate marker for *CCND1* gain at different levels of expression. A cutoff of 50% for cyclin D1 IHC staining was determined to provide an optimal balance between sensitivity and specificity, maximizing Youden's index. Tumors were considered cyclin D1 overexpressing if at least 2 observers reported ≥50% positive cells. Using the aggregated data on the percentage of cyclin D1 expression, evaluated independently by 3 pathologists, the ROC curve of cyclin D1 expression showed an area under the curve of 84.2%. Twenty-nine of 54 tumors (53.7%) in the "sequencing" cohort were classified as cyclin D1 overexpressing. Table 2 shows the agreement between *CCND1* gain and cyclin D1 IHC expression using a cutoff threshold of at least 2 raters scoring ≥50% positive tumor cells. In the "overall" cohort, 66 of 139 (47.5%) of VSCC were classified as normal and 73 of 139 (52.5%) as showing overexpression of cyclin D1.

The interobserver agreement for cyclin D1 evaluation was substantial (Fleiss' Kappa coefficient, 0.607).

CCND1/Cyclin D1 Alterations and Cliniopathological Features

Table 3 shows the main characteristics of patients in both the "sequencing" and "overall" cohorts, stratified by *CCND1* status and cyclin D1 IHC expression, respectively. No significant differences were found between the groups in either cohort in terms of age at diagnosis, tumor size, depth of invasion, lymphovascular invasion, or surgical margins. Lymph node metastases were more frequent in tumors overexpressing cyclin D1 ($P = .026$). Advanced FIGO stages were more commonly observed in patients with *CCND1* gains ($P = .011$ in the "sequencing" cohort) and in those with cyclin D1 overexpression ($P = .007$ in the "overall" cohort). Both *CCND1* gain and cyclin D1 IHC alterations were significantly associated with *TP53* mutations ($P = .040$) and abnormal p53 IHC patterns ($P = .001$).

CCND1 Gain/Cyclin D1 Overexpression and Recurrence-Free Survival

In the "sequencing" cohort, recurrence was observed in 13 (72.2%) of the 18 women with *CCND1*-gained tumors and 13 (36.1%) of the 36 women with *CCND1*-normal tumors ($P = .012$). In the "overall" cohort, 33 of 73 (45.2%) patients with

Table 3
Clinic-pathological characteristics of the patients with human papillomavirus-independent vulvar squamous cell carcinoma included in the "sequencing" cohort (*CCND1* gains determined by sequencing) and in the "overall" cohort (cyclin D1 overexpression determined by ≥50% of positive tumoral cells in the cyclin D1 immunohistochemistry)

Clinic-pathological variable	"Sequencing" cohort ($n = 54$)			"Overall" cohort ($n = 139$)		
	CCND1			Cyclin D1		
	Normal ($n = 36$)	Abnormal (gains) ($n = 18$)	*P*	Normal ($n = 66$)	Abnormal (overexpression) ($n = 73$)	*P*
Age (y)	79.6 (22.6-92.2)	77.7 (46.8-94.7)	.811	77.4 (22.6-95.8)	77.7 (30.8-95.4)	.903
Tumor size (mm)	30.0 (5.0-100.0)	31.3 (6.0-57.0)	.607	25.0 (2.0-75.0)	30.0 (4.0-100.0)	.104
Depth of invasion (mm)	5.5 (0.3-15.0)	6.2 (1.0-13.0)	.353	5.0 (0.1-35.0)	7.0 (0.3-28.0)	.082
Lymphovascular invasion	11 (30.6%)	4 (22.2%)	.519	9 (13.6%)	12 (16.4%)	.645
Affected surgical margin	1 (2.8%)	0 (0.0%)	>.999	1 (1.5%)	5 (6.8%)	.212
Lymph node metastases	15 (41.7%)	12 (66.7%)	.083	17 (25.8%)	32 (43.8%)	.026[a]
FIGO stage			.011[a]			.007[a]
Early (I/II)	25 (69.4%)	6 (33.3%)		52 (78.8%)	42 (57.5%)	
Advanced (III/IV)	11 (30.6%)	12 (66.7%)		14 (21.2%)	31 (42.5%)	
p53 status (IHC)			.010[a]			.001[a]
Normal	11 (30.6%)	0 (0.0%)		15 (22.7%)	3 (4.1%)	
Abnormal	25 (69.4%)	18 (100.0%)		51 (77.3%)	70 (95.9%)	
TP53 status (sequencing)			.040[a]			.011[a]
Wild-type	12 (33.3%)	1 (5.6%)		10 (40.0%)	3 (10.3%)	
Mutated	24 (66.7%)	17 (94.4%)		15 (60.0%)	26 (89.7%)	
Not determined	–	–		41	44	

The values show median and (range) and absolute number and (percentage).
FIGO, International Federation of Gynecology and Obstetrics; IHC, immunohistochemistry.
[a] Statistically significant differences.

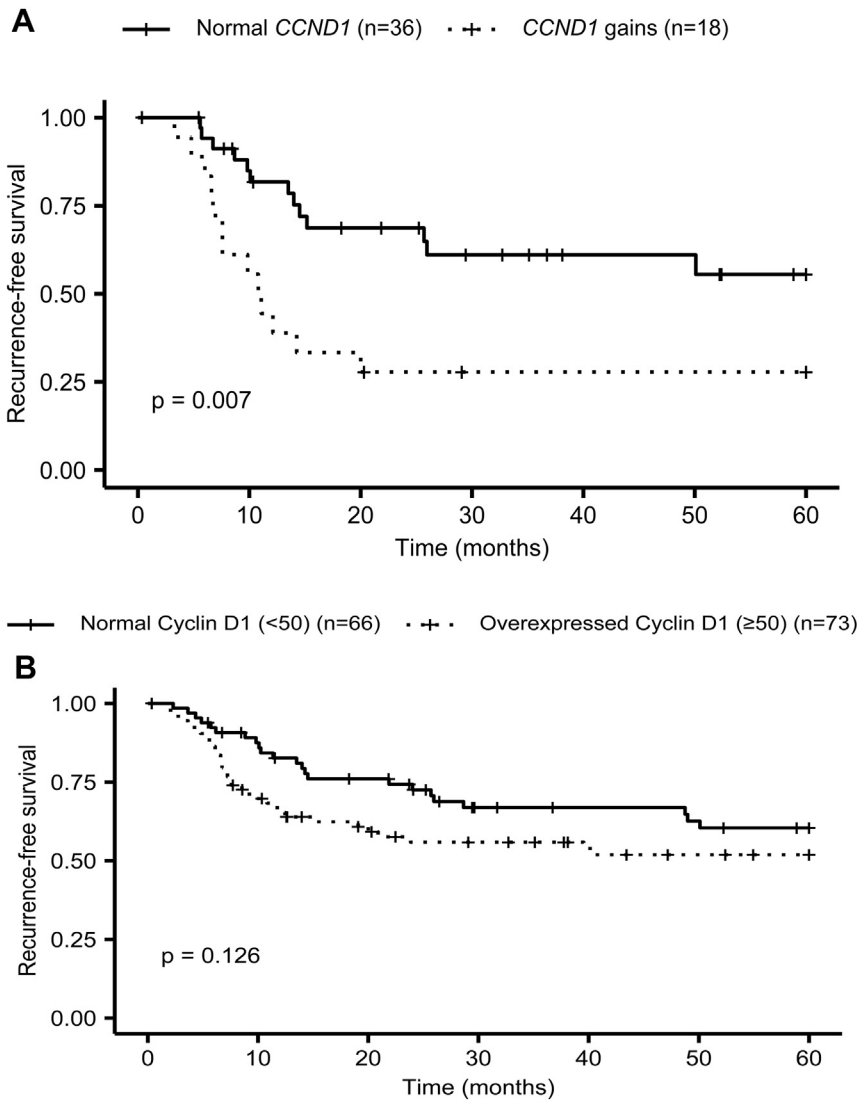

Figure 2.
Kaplan-Meier curves for recurrence-free survival in the "sequencing" cohort and the "overall" cohort, according to *CCND1* copy number status and cyclin D1 immunohistochemistry, respectively.

cyclin D1 IHC overexpression and 23 of 66 (34.8%) patients with normal cyclin D1 IHC showed recurrence ($P = .214$). Figure 2 shows the Kaplan-Meier curves for recurrence-free survival. The log-rank test revealed a significant association between *CCND1* gain and impaired recurrence-free survival ($P = .007$), with no statistically significant difference in cyclin D1 overexpression ($P = .130$). When the analysis was restricted to the patients with mutated *TP53* (sequencing cohort), the log-rank test revealed a significant association between *CCND1* gains and impaired recurrence-free survival ($P < .05$). In the overall cohort, although a trend toward higher recurrence was observed, the differences did not reach statistical significance ($P = .150$) (Supplementary Fig. S1).

Table 4 outlines the bivariate analysis after adjusting for FIGO stage and the multivariate analysis for recurrence-free survival in the "sequencing" and "overall" cohorts. Only advanced FIGO staging was associated with impaired recurrence-free survival in the multivariate analysis in both cohorts.

CCND1 Gain/Cyclin D1 Overexpression and Disease-Specific Survival

The mortality rate in the "sequencing" cohort was 55.6% (10/18) in patients with *CCND1*-gained tumors and 8.3% (3/36) in patients with *CCND1*-normal tumors ($P < .001$). In the "overall" cohort, the mortality rate was 28.8% (21/73) in patients with VSCC with cyclin D1 IHC overexpression and 4.5% (3/66) in patients with normal cyclin D1 IHC expression ($P < 0.001$). Figure 3 shows the Kaplan-Meier curves for DSS. The log-rank test showed a strong association between *CCND1* gain, cyclin D1 IHC overexpression, and DSS ($P < .001$ in both cohorts). When the analysis was restricted to the patients with mutated *TP53* (sequencing cohort) or abnormal p53 IHC, the log-rank test revealed a significant association between *CCND1* gains or cyclin D1 expression and impaired disease-specific mortality ($P = .004$ in the sequencing cohort, $P = .001$ in the overall cohort) (Supplementary Fig. S2).

Table 4
Bivariate analysis after adjusting the results for FIGO staging and multivariable analysis for recurrence-free survival of women with human papillomavirus-independent vulvar squamous cell carcinoma

Clinic-pathological variable	"Sequencing" cohort ($n = 54$)						"Overall" cohort ($n = 139$)					
	Bivariate analysis (Adjusted for FIGO staging)			Multivariate analysis			Bivariate analysis (Adjusted for FIGO staging)			Multivariate analysis		
	HR	(95% CI)	*P*	HR	(95% CI)	*P*	HR	(95% CI)	*P*	HR	(95% CI)	*P*
Age >70 y												
No	1			–			1			–		
Yes	0.58	(0.22-1.61)	0.283	–	–	–	0.95	(0.53-1.79)	0.876	–	–	–
Tumor size > 25 mm												
No	1			–			1			–		
Yes	0.86	(0.39-1.90)	0.699	–	–	–	1.10	(0.61-1.99)	0.744	–	–	–
Depth of invasion >5 mm												
No	1			–			1			–		
Yes	0.87	(0.35-2.20)	0.759	–	–	–	1.24	(0.70-2.22)	0.460	–	–	–
Lymphovascular invasion												
No	1			–			1			–		
Yes	1.09	(0.45-2.48)	0.840	–	–	–	0.90	(0.40-1.84)	0.0.773	–	–	–
Surgical margins												
Free	1			–			1			–		
Affected	0.86	(0.01-6.57)	0.913	–	–	–	0.94	(0.19-2.75)	0.924	–	–	–
Lymph node metastases												
No	1			–			1			–		
Yes	4.18	(0.78-15.4)	0.087	–	–	–	4.06	(0.82-12.4)	0.078	–	–	–
FIGO 2021 stage												
Initial (I-II)				1						1		
Advanced (III-IV)	NA	NA	NA	2.55	(1.16-5.87)	0.020[a]	NA	NA	NA	1.94	(1.13-3.31)	0.018[a]
CCND1 (sequencing)												
Normal	1			1			NA[b]			NA[b]		
Amplified	2.17	(0.98-4.81)	0.057	1.95	(0.88-4.34)	0.098	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]
Cyclin D1 (immunohistochemistry ≥50% of positive tumor cells)												
Normal	1			NA[b]			1			1		
Overexpressed	1.56	(0.72-3.52)	0.262	NA[c]	NA[c]	NA[c]	1.34	(0.79-2.33)	0.277	1.30	(0.76-2.28)	0.339
TP53 status (sequencing)												
Wild-type	1			1			NA[b]			NA[b]		
Mutated	2.31	(0.85-8.66)	0.108	2.06	(0.74-7.77)	0.177	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]
p53 Status (immunohistochemistry)												
Normal	1			NA[c]			1			1		
Abnormal	3.19	(1.02-15.9)	0.045[a]	NA[c]	NA[c]	NA[c]	1.26	(0.60-3.15)	0.571	1.16	(0.54-2.96)	0.717

Results are shown for the "sequencing" cohort" (*CCND1* gains determined by sequencing) and the "overall" cohort (cyclin D1 overexpression determined by ≥50% of positive cells in cyclin D1 IHC).
HR, hazard ratio; FIGO International Federation of Obstetrics and Gynecology; IHC, immunohistochemistry; NA, not applicable (analysis adjusted for this parameter).
[a] Values are significant.
[b] NA, not applicable (parameter not evaluated in the overall cohort).
[c] NA, not applicable due to the collinearity of sequencing and IHC variables (*CCND1* and cyclin D1, *TP53* and p53).

Table 5 shows the bivariate, FIGO stage-adjusted model, and the multivariate analysis for DSS in the "sequencing" and the "overall" cohorts. In the "sequencing" cohort, *CCND1* gains, cyclin D1 IHC overexpression, and abnormal p53 IHC were significantly associated with impaired DSS in the FIGO stage-adjusted model, but *CCND1* gain was the only factor associated with worse DSS in the multivariate analysis ($P = .014$). In the "overall" cohort, only abnormal cyclin D1 and p53 IHC statuses were shown to be significant. However, in the multivariate model, only advanced FIGO stage ($P = .032$) and cyclin D1 overexpression ($P = .001$) were associated with a worse DSS. This strong association between cyclin D1 overexpression and impaired DSS in the multivariate model remained for each rater despite the differences between them and for the mean of the 3 raters (data not shown).

Discussion

Our results show that *CCND1* gain is a frequent finding in patients with HPV-independent VSCC (affecting 33.3% of the tumors) and confers poor prognosis. Moreover, cyclin D1 IHC overexpression is a reasonable surrogate marker for *CCND1* gain and shows a similar association with an impaired prognosis. The presence of *CCND1* gains and cyclin D1 overexpression showed a strong association with impaired DSS in the multivariate analysis, and this association was much stronger than that of *TP53* mutation or p53 abnormal IHC, the significance of which was lost after adjusting for multiple variables. This is the first study comprising a large number of cases evaluating and confirming the prognostic implications of *CCND1* gains and cyclin D1 IHC overexpression in patients with VSCC.

Limited data are available on *CCND1* gain and cyclin D1 overexpression in VSCC, with reported frequencies ranging from 3% to 22%.[17,20,22,23,33-35] We observed *CCND1* gains in 33% of patients with VSCC, which was slightly higher than that previously reported. These differences could be justified, in part, by the fact that most of the studies assessing *CCND1* gains (or cyclin D1 overexpression) in VSCC included HPV-associated and HPV-independent tumors, whereas in our study, we selected

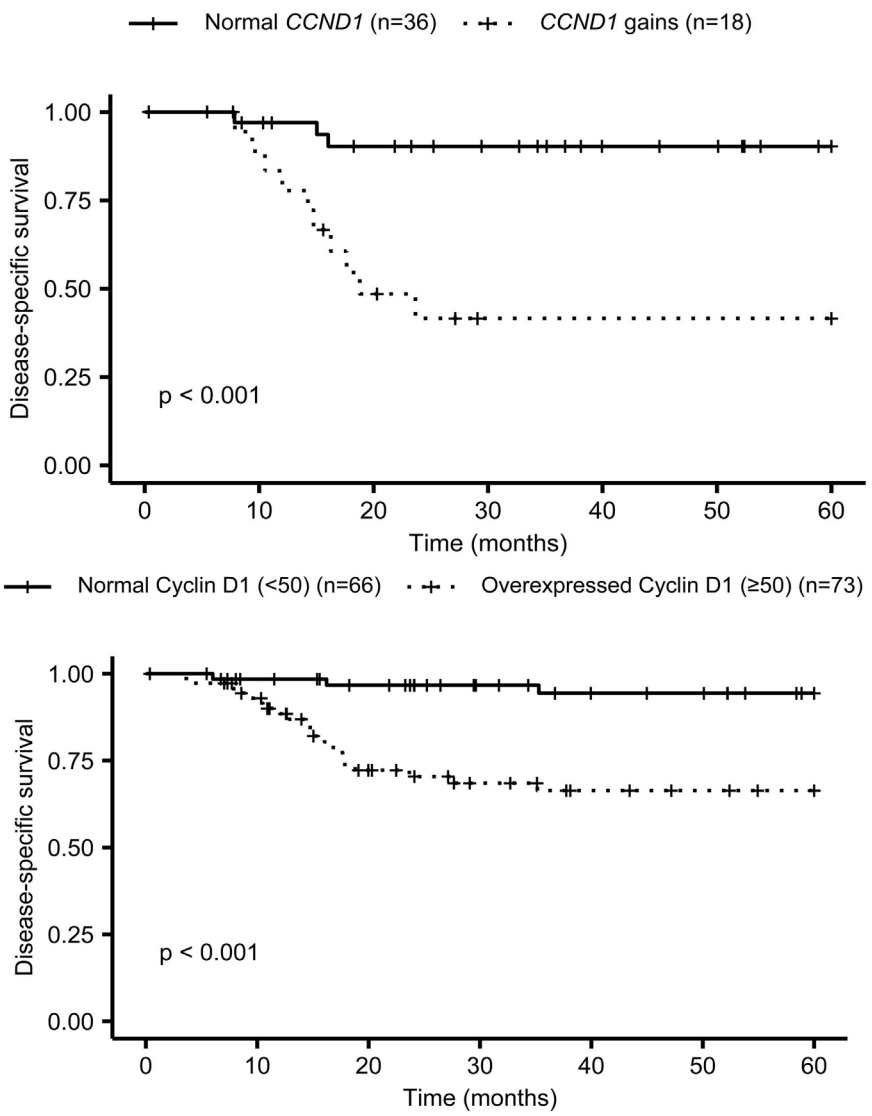

Figure 3.
Kaplan-Meier curves for disease-specific survival in the "sequencing" cohort and the "overall" cohort, according to *CCND1* copy number status and cyclin D1 immunohistochemistry, respectively.

exclusively HPV-independent tumors, in which higher rates of *CCND1* gains and cyclin D1 IHC overexpression have been described.[17,19,20,23,36] In addition, the heterogeneity in the methodologies used to evaluate cyclin D1 IHC across studies[17,20,33,34,36] may also play a role in the differences observed among the studies. In the "overall" cohort, cyclin D1 IHC overexpression was identified in 47% of patients. However, it should be emphasized that the criteria for defining cyclin D1 overexpression are markedly heterogeneous in different previously published studies, ranging from 5% to 50% of positive cells.[20,21,37,38] Moreover, several studies have considered staining patterns (nuclear and cytoplasmic staining) or intensity of staining as part of the evaluation of cyclin D1 IHC in VSCC.[36,38] In our study, different thresholds for cyclin D1 staining were carefully evaluated by comparing the percentage of cells with positive IHC staining with the status of *CCND1*. Interobserver agreement was substantial, further supporting the reliability of this technique. Cyclin D1 overexpression strongly correlated with *CCND1* gain (94% sensitivity and 67% specificity). These results were similar to those reported by Choschzick et al[20] (sensitivity, 73.7%; specificity, 64.5%). The percentage of tumors with cyclin D1 IHC overexpression that did not show *CCND1* gain was very similar in the 2 studies (41.4% in our series and 35.5% in the series by Choschzick et al[20]). This finding could be explained by the presence of additional mechanisms involved in cyclin D1 overexpression such as *TP53* mutations[36] or transcriptional activation by growth factors.[39] Finally, cyclin D1 IHC was evaluated independently by 3 pathologists, ensuring robust and reproducible assessment with substantial agreement between the observers (kappa value = 0.607).

Choschzick et al[20] reported that both *CCND1* gain and cyclin D1 IHC overexpression are associated with lymph node metastasis. In our cohort, cyclin D1 overexpression was also associated with lymph node metastasis, and patients with tumors showing *CCND1* gain or cyclin D1 overexpression were frequently diagnosed at advanced FIGO stages. However, an association with advanced FIGO stage has not been reported in other studies.[20,21,38]

The most striking finding of our study was that the association of *CCND1* gain and cyclin D1 IHC overexpression with DSS was much

Table 5
Bivariate analysis after adjusting the results for FIGO staging, and multivariable analysis for disease-specific survival of women with human papillomavirus-independent vulvar squamous cell carcinoma

Clinic-pathological variable	"Sequencing" cohort ($n = 54$)						"Overall" cohort ($n = 139$)					
	Bivariate analysis (Adjusted for FIGO staging)			Multivariate analysis			Bivariate analysis (Adjusted for FIGO staging)			Multivariate analysis		
	HR	(95% CI)	*P*	HR	(95% CI)	*P*	HR	(95% CI)	*P*	HR	(95% CI)	*P*
Age >70 y												
No	1			–			1			–		
Yes	0.46	(0.14-1.75)	.241	–	–	–	0.63	(0.28-1.57)	.310	–	–	–
Tumor size > 25 mm												
No	1			–			1			–		
Yes	1.12	(0.38-3.63)	.837	–	–	–	1.12	(0.46-2.81)	.798	–	–	–
Depth of invasion >5 mm												
No	1			–			1			–		
Yes	1.16	(0.35-4.49)	.817	–	–	–	1.50	(0.62-3.82)	.369	–	–	–
Lymphovascular invasion												
No	1			–			1			–		
Yes	0.71	(0.18-2.29)	.586	–	–	–	0.62	(0.16-1.83)	.405	–	–	–
Surgical margins												
Free	1			–			1			–		
Affected	3.90	(0.03-41.1)	.447	–	–	–	1.51	(0.17-5.82)	.647	–	–	–
Lymph node metastases												
No	1			–			1			–		
Yes	7.03	(0.67-43.6)	.091	–	–	–	7.67	(0.82-32.9)		–	–	–
FIGO 2021 stage												
Initial (I-II)				1						1		
Advanced (III-IV)	NA	NA	NA	2.64	(0.87-9.25)	0.087	NA	NA	NA	2.41	(1.08-5.39)	.032[a]
CCND1 (sequencing)												
Normal	1			1								
Amplified	5.43	(1.68-22.3)	.004[a]	4.15	(1.31-16.8)	.014[a]	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]	
Cyclin D1 (Immunohistochemistry ≥50% of positive tumor cells)												
Normal	1						1			1		
Overexpressed	26.4	(3.48-3389)	<.001[a]	NA[c]	NA[c]	NA[b]	5.86	(2.11-22.2)	<.001[a]	4.89	(1.77-18.5)	.001[a]
TP53 (sequencing)												
Wild-type	1			1			NA					
Mutated	8.59	(1.13-1102)	.034[a]	5.92	(0.75-765)	0.107	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]
p53 (Immunohistochemistry)												
Normal	1						1			1		
Abnormal	7.44	(0.98-953)	.053	NA[c]	NA[c]	NA[c]	8.11	(1.13-1030)	.033[a]	5.06	(0.68-647)	.138

Results are shown for the "sequencing" cohort (*CCND1* gains determined by sequencing) and the "overall" cohort (cyclin D1 overexpression determined by ≥50% of positive cells in the cyclin D1 immunohistochemistry).
FIGO, International Federation of Obstetrics and Gynecology; HR, hazard ratio; NA, not applicable (analysis adjusted for this parameter).
[a] Values are significant.
[b] NA, not applicable (parameter not evaluated in the overall cohort).
[c] NA, not applicable due to the collinearity of sequencing and IHC variables (*CCND1* and Cyclin D1, *TP53*, and p53).

stronger than that of *TP53* mutation or p53 abnormal IHC expression. Several recent studies have reported a strong association between *TP53* mutation or abnormal p53 IHC, a frequent finding in HPV-independent VSCC, and recurrence-free survival and DSS, suggesting that *TP53* status and/or p53 IHC expression should be included in the World Health Organization classification to further characterize HPV-independent VSCC.[6,7,9,28] Our study suggests that *CCND1* status and cyclin D1 IHC may be more relevant than *TP53*/p53 status. Indeed, as shown in a recent study conducted by our group, almost all adverse outcomes occurred in patients with *TP53* mutated or p53 IHC abnormal tumors harboring *CCND1* gain.[24]

We observed a significant association between *TP53*/p53 alterations and *CCND1* gains/cyclin D1 overexpression. Moreover, the multivariate analysis showed that *CCND1* gain/cyclin D1 overexpression is a prognostic factor independent of *TP53*/p53 status, and that abnormal *CCND1*/cyclin D1 has a significant impact when the analysis is restricted to patients with tumors with *TP53*/p53 alterations. This link between *TP53*/p53 and *CCND1*/cyclin D1 alterations has also been reported in tumors of the head and neck,[40] lung,[41] and breast,[15] and there is evidence of a regulatory interaction between the *TP53*/p53 and *CCND1*/cyclin D1 pathways, which may explain why these alterations often co-occur in aggressive tumors. Although the exact relationship may vary across tumor types, several studies show that the *TP53*/p53 and *CCND1*/cyclin D1 pathways converge at the regulation of the G1/S cell cycle transition. Moreover, some studies suggest that loss of p53 function may indirectly lead to cyclin D1 overexpression, further contributing to unchecked cellular proliferation.[42]

To the best of our knowledge, this is the first study to confirm the prognostic role of *CCND1* gain and cyclin D1 IHC overexpression in patients with VSCC. In contrast to the results of our study, previous studies have not identified an association between cyclin D1 IHC overexpression and prognosis in patients with VSCC.[20,21] Cyclin D1 overexpression has been shown to have prognostic implications

in other cancers, such as breast cancer,[43] thyroid cancer,[44] and squamous cell carcinomas of the head and neck.[45,46] However, the prognostic role of cyclin D1 in VSCC is poorly explored.[12] Some authors have suggested that cyclin D1 contributes to malignant transformation of premalignant vulvar squamous lesions.[37] Indeed, variable prevalence of cyclin D1 IHC overexpression has been described in vulvar intraepithelial neoplasia lesions (18%-30%)[37,38] and lichen sclerosus (0%-50%).[19,38]

The present study has several strengths. First, this study evaluated the prognostic relevance of cyclin D1 IHC, an area with limited literature on VSCC. Second, the large sample size and long follow-up period available for all patients allowed for accurate prognostic information. Third, the validation of cyclin D1 IHC against *CCND1* gains in a subset of patients ("sequencing" cohort) provided relevant data on the correlation between the 2 techniques. The main limitation of the present study was its retrospective nature, which may have constrained the robustness of the survival analysis. Second, due to the low prevalence of VSCC, the recruitment period was very long (48 years), and the clinical management of the cases underwent significant changes during this period. To assess this bias, we confirmed that the proportion of patients treated within each time frame was consistent across study groups (data not shown). Another potential limitation of this study is that it evaluates a relatively infrequent event (death related to VSCC), which results in wide CIs in bivariate analysis when applying Firth's penalized likelihood. Finally, in the absence of a universally endorsed pattern or threshold to define cyclin D1 IHC overexpression, we established a threshold through ROC analysis; however, the criteria used in our study diverged from those used by other researchers. This variability in cyclin D1 IHC interpretation across studies introduces substantial heterogeneity and results in difficult interpretation of different reports.

In conclusion, although they may not be entirely equivalent, cyclin D1 IHC overexpression is a valid surrogate marker for *CCND1* gain and has a strong adverse impact on the prognosis of patients with HPV-independent VSCC. This association seems to be stronger than the association with *TP53* mutation or abnormal p53 IHC. Further research is needed to confirm this association and to elucidate whether changes in the clinical management of patients with VSCC might be warranted. With standardized evaluation methods, cyclin D1 IHC would have potential as a predictive biomarker in patients with VSCC.

Author Contributions

N.C.-D. performed study design, patients' follow-up and collection of clinical data, investigation, and writing of first draft. O.O. performed study design, data curation and analysis, investigation, and writing of first draft. N.P. performed data analysis, writing of first draft, investigation, and study supervision. M.d.P. performed statistical analysis, patients' follow-up and collection of clinical data. L.D.-A. performed writing and editing. L.S. and K.D. performed investigation and editing of first draft. L.M. and N.V. were involved in laboratory analyses, investigation, and editing of first draft. A.T. contributed to investigation, resources, supervision, patients' follow-up, and collection of clinical data. A.S. contributed to investigation and interpretation of laboratory analyses. R.A. performed bioinformatics analysis, investigation, and editing of first draft. L.G. was involved in resources, patients' follow-up and collection of clinical data, investigation, and editing of first draft. N.R. contributed to resources, supervision, study conceptualization, data curation, bioinformatics analysis, and editing of first draft.

Data Availability

All data supporting the findings of this study are available within the article, in the supplementary data files, or from the corresponding author (natalia.rakislova@isglobal.org). All custom code used in this study is also available from the corresponding author upon reasonable request.

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Declaration of Competing Interest

The authors have declared that no conflict of interest exists.

Ethics Approval and Consent to Participate

Institutional ethical approval for this study was obtained in 2020 (registry reference HCB/2020/1198). All participating patients provided written informed consent in accordance with institutional and legal regulations.

Supplementary Material

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